

## S-Nitrosation of $\beta$ -Catenin and p120 Catenin : A Novel Regulatory Mechanism in Endothelial Hyperpermeability

Natalie Marín, Patricia Zamorano, Rodrigo Carrasco, Patricio Mujica, Francisco G. González, Claudia Quezada, Cynthia J. Meininger, Mauricio P. Boric, Walter N. Durán and Fabiola A. Sánchez

*Circ Res.* 2012;111:553-563; originally published online July 9, 2012;

doi: 10.1161/CIRCRESAHA.112.274548

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2012 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circres.ahajournals.org/content/111/5/553>

Data Supplement (unedited) at:

<http://circres.ahajournals.org/content/suppl/2012/07/09/CIRCRESAHA.112.274548.DC1.html>

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

**Reprints:** Information about reprints can be found online at:  
<http://www.lww.com/reprints>

**Subscriptions:** Information about subscribing to *Circulation Research* is online at:  
<http://circres.ahajournals.org/subscriptions/>

## S-Nitrosation of $\beta$ -Catenin and p120 Catenin A Novel Regulatory Mechanism in Endothelial Hyperpermeability

Natalie Marín,\* Patricia Zamorano,\* Rodrigo Carrasco, Patricio Mujica, Francisco G. González, Claudia Quezada, Cynthia J. Meininger, Mauricio P. Boric, Walter N. Durán, Fabiola A. Sánchez

**Rationale:** Endothelial adherens junction proteins constitute an important element in the control of microvascular permeability. Platelet-activating factor (PAF) increases permeability to macromolecules via translocation of endothelial nitric oxide synthase (eNOS) to cytosol and stimulation of eNOS-derived nitric oxide signaling cascade. The mechanisms by which nitric oxide signaling regulates permeability at adherens junctions are still incompletely understood.

**Objective:** We explored the hypothesis that PAF stimulates hyperpermeability via S-nitrosation (SNO) of adherens junction proteins.

**Methods and Results:** We measured PAF-stimulated SNO of  $\beta$ -catenin and p120-catenin (p120) in 3 cell lines: ECV-eNOSGFP, EAhy926 (derived from human umbilical vein), and postcapillary venular endothelial cells (derived from bovine heart endothelium) and in the mouse cremaster muscle in vivo. SNO correlated with diminished abundance of  $\beta$ -catenin and p120 at the adherens junction and with hyperpermeability. Tumor necrosis factor- $\alpha$  increased nitric oxide production and caused similar increase in SNO as PAF. To ascertain the importance of eNOS subcellular location in this process, we used ECV-304 cells transfected with cytosolic eNOS (GFPeNOSG2A) and plasma membrane eNOS (GFPeNOSCAAX). PAF induced SNO of  $\beta$ -catenin and p120 and significantly diminished association between these proteins in cells with cytosolic eNOS but not in cells wherein eNOS is anchored to the cell membrane. Inhibitors of nitric oxide production and of SNO blocked PAF-induced SNO and hyperpermeability, whereas inhibition of the cGMP pathway had no effect. Mass spectrometry analysis of purified p120 identified cysteine 579 as the main S-nitrosated residue in the region that putatively interacts with vascular endothelial-cadherin.

**Conclusions:** Our results demonstrate that agonist-induced SNO contributes to junctional membrane protein changes that enhance endothelial permeability. (*Circ Res.* 2012;111:553-563.)

**Key Words:** S-nitrosation ■ adherens junction ■ microvascular permeability ■ nitric oxide ■ p120

Cell life depends on an adequate supply of nutrients and removal of catabolites via blood circulation. This important exchange function is controlled mainly by the microvascular endothelium. Inflammation and wound healing require specific regulation of microvascular permeability to macromolecules to enable tissue repair. The regulation of permeability occurs primarily at postcapillary venules in vivo through activation of endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO), which has been identified as a key signaling element in eliciting hyperperme-

ability.<sup>1-5</sup> The mechanisms by which eNOS-derived NO induces hyperpermeability include the internalization of eNOS indicating that eNOS location and exact delivery of NO regulate the development of hyperpermeability.<sup>2,6</sup>

Endothelial cells from postcapillary venules display adherens junctions, which contain vascular endothelial (VE)-cadherin, forming a complex with the cytosolic proteins  $\alpha$ -catenin,  $\beta$ -catenin, plakoglobin, and p120-catenin (p120).<sup>7-11</sup> Proinflammatory agents stimulate signaling cascades that by phosphorylation of adherens junction

Original received May 28, 2012; revision received June 28, 2012; accepted July 9, 2012. In June 2012, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.35 days.

From the Instituto de Inmunología (N.M., P.Z., R.C., F.A.S.) and Bioquímica (C.Q.), Universidad Austral de Chile, Valdivia, Chile; Department of Systems Biology and Translational Medicine (C.J.M.), Texas A&M Health Science Center, Temple, TX; Departamento de Fisiología (F.G.G., M.P.B.), Pontificia Universidad Católica de Chile, Santiago, Chile; and Department of Pharmacology and Physiology (P.M., F.G.G., W.N.D.), University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, NJ.

\*These authors contributed equally to this work.

The online-only Data Supplement is available with this article at <http://circres.ahajournals.org/lookup/suppl/10.1161/CIRCRESAHA.112.274548/-DC1>.

Correspondence to Fabiola Sánchez, Instituto de Inmunología, Universidad Austral de Chile, Los Laureles s/n, 511-0566, Valdivia, Chile. E-mail [fabiolasanchez@uach.cl](mailto:fabiolasanchez@uach.cl)

© 2012 American Heart Association, Inc.

*Circulation Research* is available at <http://circres.ahajournals.org>

DOI: 10.1161/CIRCRESAHA.112.274548

**Non-standard Abbreviations and Acronyms**

<b>CVEC</b>	bovine coronary venular postcapillary endothelial cells
<b>eNOS</b>	endothelial nitric oxide synthase
<b>L-NMA</b>	L-NG-methyl-L-arginine
<b>NAC</b>	N-acetyl-L-cysteine
<b>NO</b>	nitric oxide
<b>ODQ</b>	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
<b>PAF</b>	platelet activating factor
<b>PKG</b>	protein kinase G
<b>sGC</b>	soluble guanylate cyclase
<b>SNO</b>	S-nitrosation
<b>TNF-<math>\alpha</math></b>	Tumor necrosis factor $\alpha$
<b>VE</b>	vascular endothelial

proteins lead to their internalization, which results in increased endothelial paracellular permeability.<sup>12,13</sup>

Despite advances in the research of hyperpermeability regulation, the mechanisms by which NO signaling influences processes occurring at the adherens junctions remain unknown. The classic dogma in NO signaling establishes that all of the actions of NO are mediated via soluble guanylate cyclase (sGC) and protein kinase G (PKG). However, increases in permeability have been described in some cellular models regardless of PKG activation but still requiring NO production.<sup>14</sup> Recently, S-nitrosation (SNO) has emerged as an important NO-dependent posttranslational modification of free-thiol cysteines that alters the function of proteins and requires proximity between eNOS and the target proteins for appropriate NO delivery.<sup>15–17</sup> We report here experiments testing the hypothesis that NO regulates endothelial permeability to macromolecules by SNO of  $\beta$ -catenin and p120, 2 important and integral components of the adherens junction. We determined that platelet-activating factor (PAF) induces SNO of  $\beta$ -catenin and p120 and their internalization away from the plasma membrane. These processes are associated with the onset of hyperpermeability. Furthermore, we demonstrate that eNOS located in the cytosol, but not the plasma membrane-anchored eNOS, causes SNO of  $\beta$ -catenin and p120. This advance in knowledge may serve as a basis for the development of therapeutic agents in the treatment of vascular diseases having a phase characterized by inflammation (eg, ischemia-reperfusion injury, stroke, and atherosclerosis).

**Methods**

An expanded Methods section is provided in the online-only Data Supplement.

**Reagents**

PAF and N-acetyl-L-cysteine (NAC) were obtained from Calbiochem. FITC-labeled dextran 70 (FITC-Dx-70; molecular weight: 70 000 Da), Acetylcholine, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), N-ethylmaleimide, and NG-methyl-L-arginine (L-NMA) were from Sigma. Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) was from Roche.

**Antibodies**

Mouse anti-p120 catenin was from BD Transduction Laboratories. Rabbit anti- $\beta$ -catenin and mouse anti- $\beta$ -actin, were from Sigma.

**Plasmids and Cell Transfections**

GFPeNOS-G2A and GFPeNOS-CAAX constructs were kindly provided by Dr. David Fulton (Georgia Health Sciences University, Augusta, GA). ECV-304 cells were transfected with fluorescently labeled eNOS mutant constructs using Lipofectamine according to manufacturers' instructions (Invitrogen). Transfected cells were selected for growth in medium including 1 mg/mL of geneticin (G418) (Invitrogen). Resistant colonies were maintained in the complete Dulbecco's modified Eagle's medium containing G418 (400  $\mu$ g/mL).

**Cell Culture**

ECV-304, ECV-eNOSGFP, ECV-GFPeNOS-G2A, ECV-GFPeNOS-CAAX, EAhy296, and postcapillary venular endothelial cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. ECV-eNOSGFP, ECVG2A-eNOSGFP, and ECVCAAX-eNOSGFP were additionally supplemented with geneticin (G418) 400  $\mu$ g/mL. ECV-304 and ECV-eNOSGFP were kindly donated by Dr. William Sessa (Yale University, New Haven, CT). EAhy926 cells were kindly provided by Dr C. J. S. Edgell (University of North Carolina, Chapel Hill, NC).

**Immunoprecipitation**

Cell lysates from control and agonist-treated cells were incubated with specific antibodies overnight at 4°C. Protein A/G beads were added to samples for 2 hours at 4°C and then pelleted by centrifugation and washed with lysis buffer. Proteins of interest were detected using Western blotting and chemiluminescence. Quantification of changes from control was evaluated by densitometric analysis of Western blots using the NIH Image J Program.

**Biotin-Switch Assay**

Proteins were denatured with sodium dodecyl sulfate in the presence of methyl methanethiosulfonate.<sup>18</sup> After acetone precipitation to remove excess methyl methanethiosulfonate, 1 mmol/L ascorbate and biotin- N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide were added to reduce the SNO bond and label the reduced thiol with biotin, respectively. Biotinylated proteins were captured with streptavidin-agarose beads and then separated by sodium dodecyl sulfate-PAGE and detected with specific antibodies. The biotin-switch assay was performed on 100  $\mu$ g of lysates from control and agonist-treated cells. Proteins of interest were detected by Western blotting.

**Immunofluorescence Microscopy**

We followed established protocols.<sup>19,20</sup> Cells were cultured on glass coverslips and treated with agonist and then fixed and permeabilized in 100% ethanol for 10 minutes at -20°C. The secondary Alexa Fluor conjugated antibodies were added after incubation with corresponding primary antibodies for 1 hour at room temperature. Coverslips were mounted and visualized by fluorescence microscopy. Images were obtained using an epifluorescence microscope (Axioscop; Carl Zeiss) equipped with a 100 $\times$ oil immersion objective lens and Axio Vision Rel software (Zeiss, Germany). Eight-bit images were prepared for illustration in Photoshop (Adobe).

**Endothelial Permeability Assay**

Monolayer permeability was determined as described previously.<sup>2,21</sup> Cells were grown on fibronectin-coated polycarbonate membranes,

for 5 to 6 days to achieve confluence. On the day of the experiment, the membranes were placed in a Navicte system (San Diego, CA). Luminal and abluminal chambers were filled with Dulbecco's modified Eagle's medium without phenol red. After a 15-minute equilibration period, the luminal chamber was loaded with FITC-Dx-70 (final concentration 13.3 mg/mL). Samples for baseline permeability were obtained every 5 minutes for a period of 30 minutes. After addition of PAF, samples were obtained for an additional 30-minute period. In the experiments using NAC (2.5 mmol/L), this agent was added for 1.5 hour to the cells in the plates prior to the addition of agonists. Fresh NAC was applied for 1 more hour to the cells when they were transferred to the Navicte system. In the experiments using L-NMA (300  $\mu$ mol/L), the NOS inhibitor was added for 1 hour while the cells were in the Navicte system before PAF addition. The permeability to FITC-Dx-70 was determined according to the Fick equation. FITC-Dx-70 was measured using a spectrofluorometer.

### In Vivo Cremaster Preparation

Male wild-type (C57BL/6J; Jackson Laboratory, Bar Harbor, MA) were anesthetized with Ketamine (90 mg/Kg)-Xylazine (10 mg/Kg). The cremaster muscle was exposed via a scrotal incision and gently separated from the subcutaneous interstitial tissue under continuous superfusion with intravital buffer solution equilibrated with 95% N<sub>2</sub> plus 5% CO<sub>2</sub>, pH 7.4, 35°C. The whole cremaster sac, containing the testis, was positioned in a plastic container and superfused. After 15 minutes equilibration, 10<sup>-7</sup> mol/L PAF was applied topically, bathing the whole muscle for 3 minutes. The cremaster and the testis were ligated and quickly excised. The muscle was immediately separated from the testis and homogenized in 500  $\mu$ L buffer containing an antiprotease cocktail. The contralateral cremaster was treated similarly, except that buffer was applied instead of PAF. Both homogenates were processed simultaneously for biotin switch, followed by streptavidin pull down and Western blotting. All experiments on animals were carried out at the P. Universidad Católica de Chile, approved by the Institutional Bioethics and Biosecurity Committee and conducted according to NIH Guidelines for the use of animals in research.

### Identification of S-Nitrosated Cysteines in p120-Catenin

The sites of p120 SNO were identified by MS/MS mass spectrometry. One  $\mu$ g recombinant p120 was treated with 100  $\mu$ mol/L S-nitrosoglutathione 37°C for 30 minutes. The protein was then precipitated 4 times with ice cold acetone (50  $\mu$ g lysozyme as a carrier) and washed with ice cold acetone. Free thiols were blocked with 20 mmol/L methyl methanethiosulfonate followed by acetone precipitation and washed with 80% ice cold acetone for 3 times. SNO cysteines were reduced with 10 mmol/L ascorbate and labeled with biotin *N*-[6-(biotinamido) hexyl]-3'-(2'-pyridyl)dithio) propionamide. The biotinylated sample (about 0.1  $\mu$ g p120) was resolved in 8 mol/L urea after acetone precipitation and diluted 10 times using 50 mmol/L NH<sub>4</sub>HCO<sub>3</sub> and 0.01  $\mu$ g trypsin was added for in solution digestion. The peptides were desalted with a C<sub>18</sub> ziptip, and identified by Orbitrap Velos MS. The MS/MS spectra were searched against a Swissprot human database using Thermo proteome Discoverer 1.3.0.339 software with biotin *N*-[6-(biotinamido) hexyl]-3'-(2'-pyridyl)dithio) propionamide as a variable modification. Peptides identified with 95% confidence interval were displayed in Scaffold3 software. Protein and peptide false discovery rates was <1%.

### Statistical Analysis

Experiments were conducted in groups with minimum n=3. Data were expressed as mean  $\pm$  standard error. Apparent differences were assessed for statistical significance using paired Student *t* test and/or the Newman-Keuls test. Significance was accepted at values of *P*<0.05.

## Results

### SNO Reduces the Integrity of the Endothelial Barrier

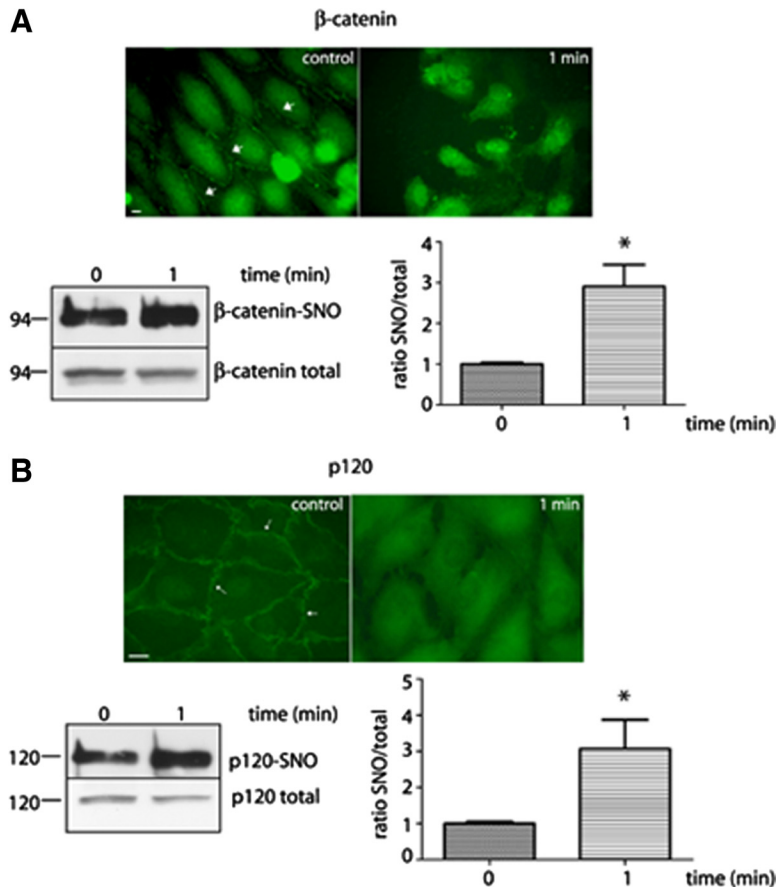
NO derived from eNOS has been largely recognized as a regulatory signal for endothelial permeability; however, the exact mechanisms by which this regulation is achieved are incompletely understood. To investigate how NO modifies proteins of the endothelial barrier to increase microvascular permeability, we applied 10<sup>-7</sup> mol/L PAF to confluent monolayers of bovine coronary venular postcapillary endothelial cells<sup>21,22</sup> and assessed whether or not PAF-induced release of NO causes SNO of junctional proteins and loss of morphological integrity of the intercellular junctions. We used postcapillary venular endothelial cells initially because these cells are derived from the main site where in vivo regulation of microvascular permeability to macromolecules occurs. We examined the impact of PAF on  $\beta$ -catenin and p120, 2 proteins that are constituents of the adherens junction. Figure 1 shows that in control conditions  $\beta$ -catenin and p120 appear as continuous belt-like structures on the plasma membrane, as revealed by indirect immunofluorescence microscopy. PAF induced significant increment of SNO of  $\beta$ -catenin and p120 as shown by representative Western blot and statistical analysis. PAF-induced SNO was associated with redistribution of these proteins, away from the plasma membrane, and the appearance of a discontinuous pattern on the cell membrane (Figure 1). The increase in SNO was significant at 1 minute after application of PAF, a time lapse that is compatible with a mechanistic causal effect, in accordance with the time course of PAF-induced hyperpermeability.<sup>2,6,21,23</sup>

### SNO Is a Widespread Regulatory Mechanism for the Reorganization of Endothelial Junctional Proteins

To test whether or not the regulation of endothelial permeability to macromolecules by SNO is a widespread mechanism, we tested PAF-induced effects in EAhy926 cells and in ECV-304 cells transfected with a "wild-type" eNOS construct (ECVeNOSGFP), in which eNOS achieves the same distribution found in native endothelial cells.<sup>23</sup> The characteristics of these cells are described in online-only Supplemental Data. In control EAhy926 cells we observed a clear label of  $\beta$ -catenin at the plasma membrane, which tended to disappear with PAF treatment. Likewise the strong fluorescence due to p120 at the plasma membrane observed in control conditions decreased as the result of PAF application (Figure 2A). A similar response pattern was observed in ECVeNOSGFP cells, ie, the immunofluorescence signal for  $\beta$ -catenin and p120 was predominantly at the cell border in control conditions (Figure 2B, arrows) and decreased after 1 minute of PAF treatment. The pattern of both proteins redistribution was maintained at least for 5 minutes during PAF treatment.

The changes in the localization of  $\beta$ -catenin and p120 were correlated with SNO. In control conditions, we detected a basal level of SNO of both proteins in EAhy926 and ECV-eNOSGFP cells (Figure 2A and 2B, lower panels). As





**Figure 1. Correlation between protein organization at the cell membrane and S-nitrosation (SNO) of  $\beta$ -catenin and p120 in postcapillary venular endothelial cells.** **A, Top:** Indirect immunofluorescence staining for  $\beta$ -catenin in control conditions and after stimulation with  $10^{-7}$  mol/L platelet-activating factor (PAF). **Arrows** indicate the presence of  $\beta$ -catenin in the cell plasma membrane. Bar represents  $10\ \mu\text{m}$ . **Bottom:** SNO of  $\beta$ -catenin as measured by biotin switch. The quantification is shown as the ratio of the S-nitrosylated  $\beta$ -catenin to the total  $\beta$ -catenin on the right lower panel.  $*P < 0.05$  compared to control;  $n = 3$ . **B, Top:** Indirect immunofluorescence staining for p120 in control conditions and after stimulation with  $10^{-7}$  mol/L PAF. **Left bottom:** SNO of p120 detected by biotin switch. The quantification is shown as the ratio of the S-nitrosylated p120 to the total p120 on the right lower panel.  $*P < 0.05$  as compared with control;  $n = 3$ .

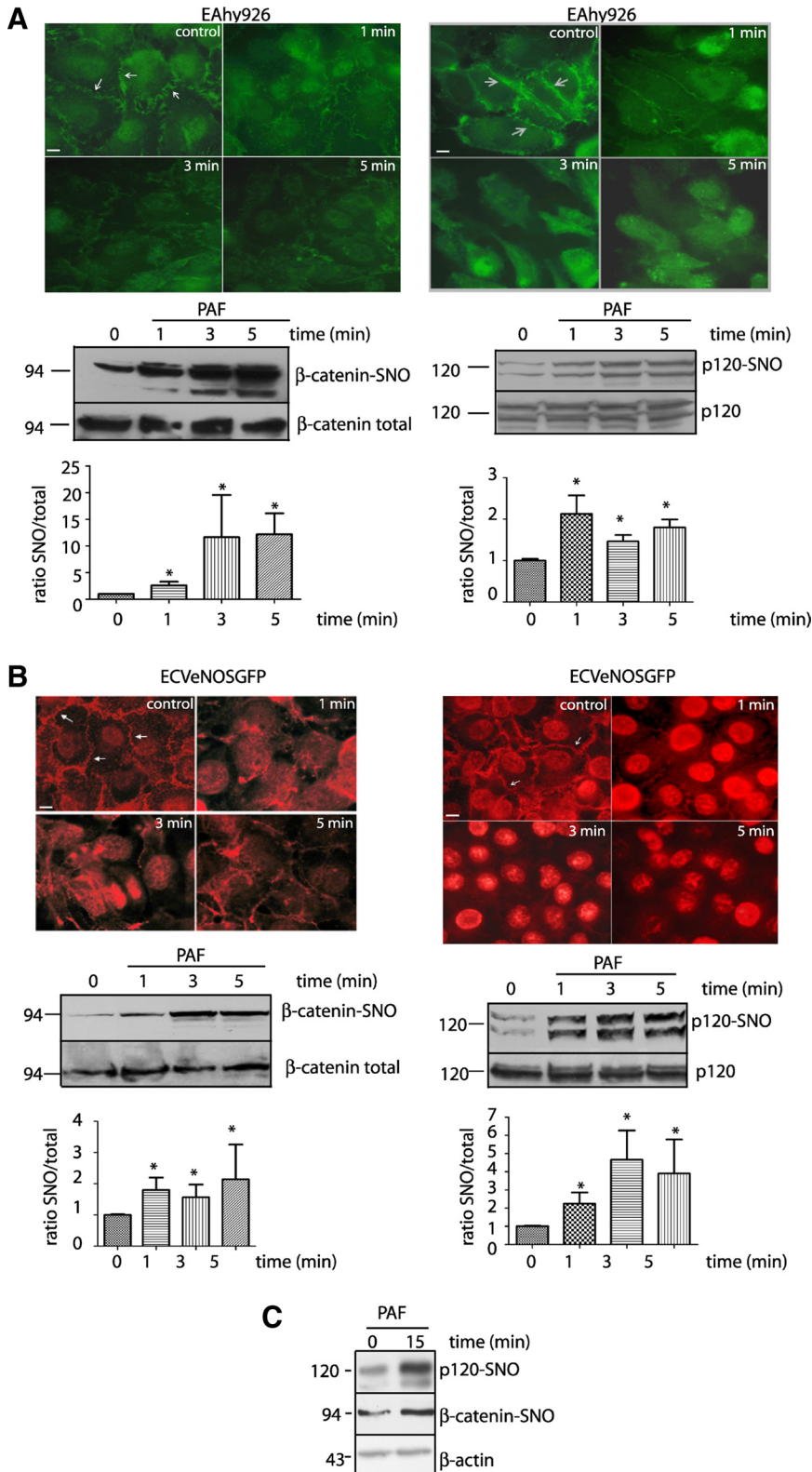
early as after 1 minute of administration, PAF induced a marked and significant increase in SNO of  $\beta$ -catenin and p120 in both cell models. PAF-induced SNO was associated with reorganization of the distribution of  $\beta$ -catenin and p120 at the plasma membrane in both cell types. Furthermore, these modifications were maintained until the end of the observation period. These observations are consistent with a regulatory association between PAF-induced SNO and PAF-induced hyperpermeability. Interestingly, SNO of  $\beta$ -catenin and p120 was observed also after 15 minutes of continuous application of PAF (Figure 2C).

To confirm that induction of SNO is a common property of proinflammatory agents, we tested whether or not tumor necrosis factor  $\alpha$ - $\alpha$  (TNF- $\alpha$ ), a well-known proinflammatory agent that increases permeability, recapitulates PAF-induced SNO. Figure 3A shows that TNF- $\alpha$  increased NO production from  $8.4 \pm 3.7$  pmoles/min to  $26.9 \pm 3.2$  pmoles/min at 1 minute after its application. At this time, TNF- $\alpha$  also increased SNO of  $\beta$ -catenin and p120 indicating that SNO is a mechanism shared among agents that cause inflammatory hyperpermeability. To evaluate whether or not release of NO induces nitrosation of junctional proteins independently of changes in permeability, we applied acetylcholine, an agonist that activates eNOS but does not increase permeability.<sup>23,24</sup> Figure 3B illustrates that acetylcholine does not enhance SNO of  $\beta$ -catenin and p120. In addition, to test whether or not nitrosation-induced hyperpermeability is associated with acti-

vation of sGC, we pretreated the cells with ODQ prior to PAF application. Figure 3C shows that PAF caused a significant increase in permeability to macromolecules in the presence of ODQ, suggesting that PAF-induced SNO of junctional proteins is sufficient to cause hyperpermeability.

### eNOS Location Determines SNO of Junctional Proteins

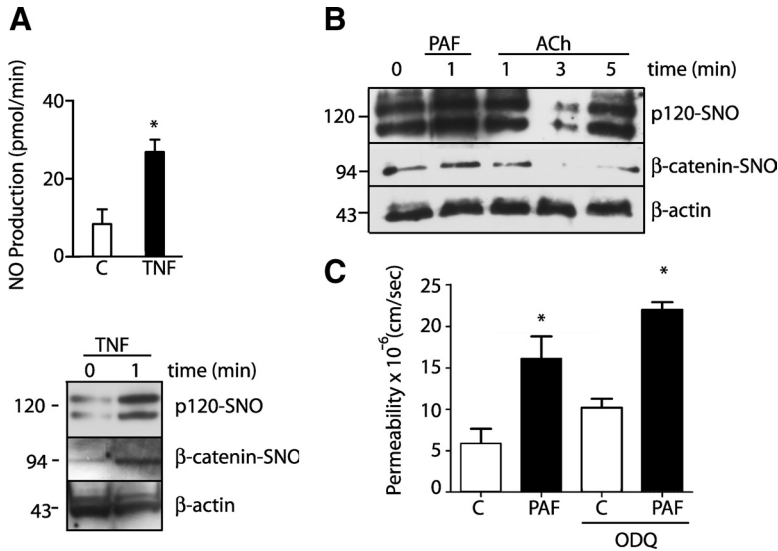
Vasoactive agents induce molecular movement or translocation of eNOS in vitro and in vivo.<sup>25</sup> Recently, we demonstrated the importance of localized NO production in hyperpermeability using ECV cells transfected with eNOS constructs that target eNOS to different subcellular locations, GFPeNOS-G2A (targets to the cytosol) and GFPeNOS-CAAX (targets to the plasma membrane).<sup>6,26,27</sup> Even though both cell lines are able to produce NO in response to PAF, only GFPeNOS-G2A, showing eNOS expression in the cytosol, was able to increase permeability in response to PAF.<sup>6</sup> On the other hand, ECV-GFPeNOS-CAAX cells, expressing eNOS at the plasma membrane, did not increase permeability in response to PAF despite producing NO at the same magnitude as ECV-GFPeNOS-G2A and ECV cells transfected with wild type eNOS. To explore whether or not SNO of junctional proteins depends on eNOS location, we tested PAF-induced SNO of  $\beta$ -catenin and p120 in ECV-GFPeNOS-G2A and ECV-GFPeNOS-CAAX cells. Figure 4 displays our results. In agreement with our hypothesis, PAF



**Figure 2. Platelet-activating factor (PAF) induces internalization and S-nitrosation (SNO) of  $\beta$ -catenin and p120 in EAhy926 cells and ECVeNOS-GFP.** **A**, Experiments in EAhy926 cells. **Top**: Indirect immunofluorescence staining of EAhy926 cells for  $\beta$ -catenin and p120 after stimulation with  $10^{-7}$  mol/L PAF. **Middle**: SNO of  $\beta$ -catenin (left side) and p120 (right side) as detected by biotin switch. **Bottom**: Bar graph showing the quantification of the SNO signal for  $\beta$ -catenin (left side) and p120 (right side).  $*P < 0.05$  compared with control;  $n = 3$ . **B**, Experiments in ECVeNOS-GFP. **Top**: Indirect immunofluorescence staining of ECVeNOS-GFP for  $\beta$ -catenin (left side) and p120 (right side) in control and at different times after stimulation with  $5 \times 10^{-7}$  mol/L PAF. **Middle**: SNO of  $\beta$ -catenin (left side) and p120 (right side) as detected by biotin switch. **Bottom**: Bar graph reporting the quantification of the SNO signal in Western blots for  $\beta$ -catenin (left side) and p120 (right side).  $*P < 0.05$  as compared with control;  $n = 3$ . **C**, Prolonged application of PAF induces SNO of  $\beta$ -catenin and p120 in EAhy926 cells. PAF at  $10^{-7}$  mol/L was applied for 15 minutes. Protein extracts were processed for biotin switch assay and probed with anti- $\beta$ -catenin and anti-p120 antibodies. Beta-actin was used as a load control. Bars in microphotographs represent  $10 \mu\text{m}$ .

stimulated SNO of  $\beta$ -catenin and p120 in cells with eNOS targeted to the cytosol (ECV-GFPeNOS-G2A; Figure 4A) but not in cells with eNOS targeted to the plasma membrane (ECV-GFPeNOS-CAAX; Figure 4B). PAF caused significant SNO of junctional proteins only in cells containing eNOS targeted to the cytosol when applied for 1, 3, and 5 minutes,

which correlates well with the PAF-induced reorganization of  $\beta$ -catenin and p120 in adherens junctions in ECV-eNOSGFP (Figure 2). Interestingly, targeting of eNOS to the plasma membrane precluded stimulation of SNO of  $\beta$ -catenin and p120 and is in agreement with our report demonstrating that PAF fails to cause hyperpermeability in these cells.<sup>6</sup>



**Figure 3. S-nitrosation (SNO) is a widespread regulatory mechanism specific for permeability and independent of sGC and protein kinase G (PKG).** **A**, TNF- $\alpha$  induces nitric oxide (NO) production and SNO of  $\beta$ -catenin and p120 in EAhy926 cells. Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) at 50 ng/mL was applied for 1 minute. Protein extracts were processed for biotin switch assay and probed with anti  $\beta$ -catenin and anti p120 antibodies. Beta-actin was used as a load control. **B**, ACh at  $10^{-5}$  moles/L does not induce SNO of  $\beta$ -catenin and p120 in EAhy926 cells. **C**, PAF at  $10^{-7}$  moles/L increases permeability to FITC-dextran-70 across confluent EAhy926 monolayers. PAF-stimulated hyperpermeability is not affected by inhibition of sGC with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). \* $P < 0.05$  compared with control;  $n = 3$ .

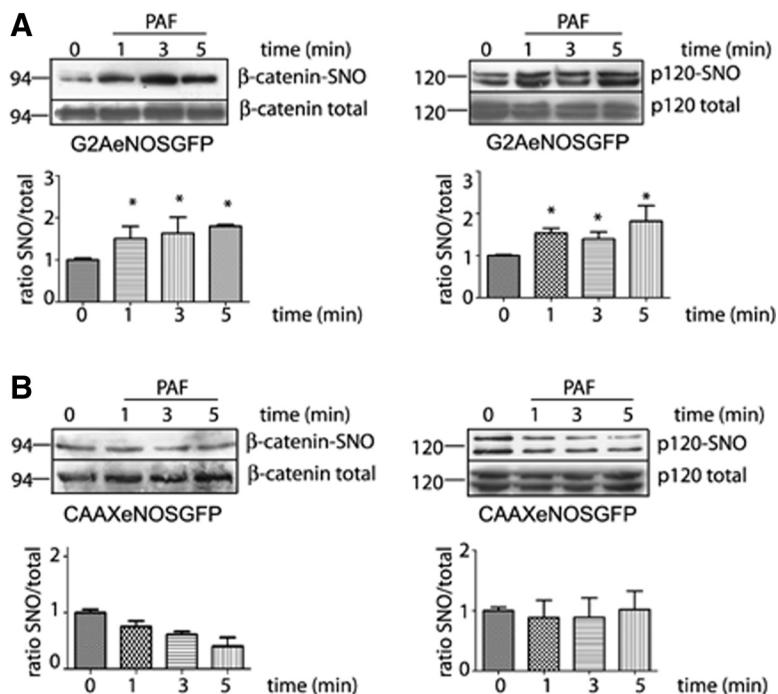
**SNO Disrupts the Association Between  $\beta$ -Catenin and p120**

We investigated whether or not PAF-induced SNO impacts on the association between  $\beta$ -catenin and p120 in ECV-GFPeNOS-G2A and ECV-GFPeNOS-CAAX cells. We tested this concept by immunoprecipitation using an antibody against  $\beta$ -catenin and probing for the presence of p120 in the precipitate. The data were quantified as a ratio to the total content of  $\beta$ -catenin in the input and compared to the levels seen at time 0. Figure 5 demonstrates that PAF significantly decreased the association between  $\beta$ -catenin and p120 in ECV-GFPeNOS-G2A cells (cytosolic eNOS cells) when applied for 1, 3, and 5 minutes (Figure 5A). Interestingly, PAF failed to alter the association between  $\beta$ -catenin and p120 in ECV-GFPeNOS-CAAX cells (plasma membrane eNOS; Figure 5B). These data confirm the importance of

eNOS location in regards to the ability of PAF to elicit NO-dependent functional reactions and support the concept that increases in permeability are related to modifications in the association between proteins that make up the junctional complex.

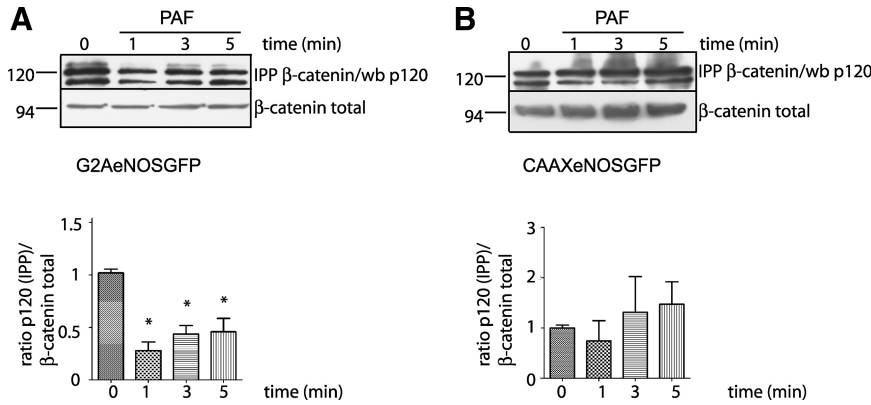
**SNO of Junctional Proteins Increases Permeability to Macromolecules**

To assess directly the cause-effect relationship between SNO and hyperpermeability to macromolecules, we measured PAF-induced transport of FITC-dextran 70 (molecular weight: 70 kDa) across confluent monolayers of EAhy926 cells under control conditions as well as under inhibition of eNOS by L-NMA (L-N-methyl arginine) and inhibition of SNO by NAC (N-acetyl-L-cysteine). Figure 6A shows that



**Figure 4. Subcellular location of endothelial nitric oxide synthase (eNOS) determines S-nitrosation (SNO) of  $\beta$ -catenin and p120.** Protein extracts from ECV-GFPeNOS-G2A and ECV-GFPeNOS-CAAX cells treated with  $5 \times 10^{-7}$  mol/L platelet-activating factor (PAF) for different times were processed for biotin switch assay and probed with anti  $\beta$ -catenin and anti p120 antibodies. **A, Top**: PAF-induced SNO of  $\beta$ -catenin and p120 in ECV-GFPeNOS-G2A cells. **Bottom**: Bar graph showing the quantification of the Western blots of  $\beta$ -catenin (left side) and p120 (right side). \* $P < 0.05$  compared to control;  $n = 3$ . **B, Top**: PAF-induced SNO of  $\beta$ -catenin and p120 in ECV-GFPeNOS-CAAX cells. **Bottom**: Bar graph showing the quantification of the Western blots of  $\beta$ -catenin (left side) and p120 (right side). \* $P < 0.05$  compared to control;  $n = 3$ .



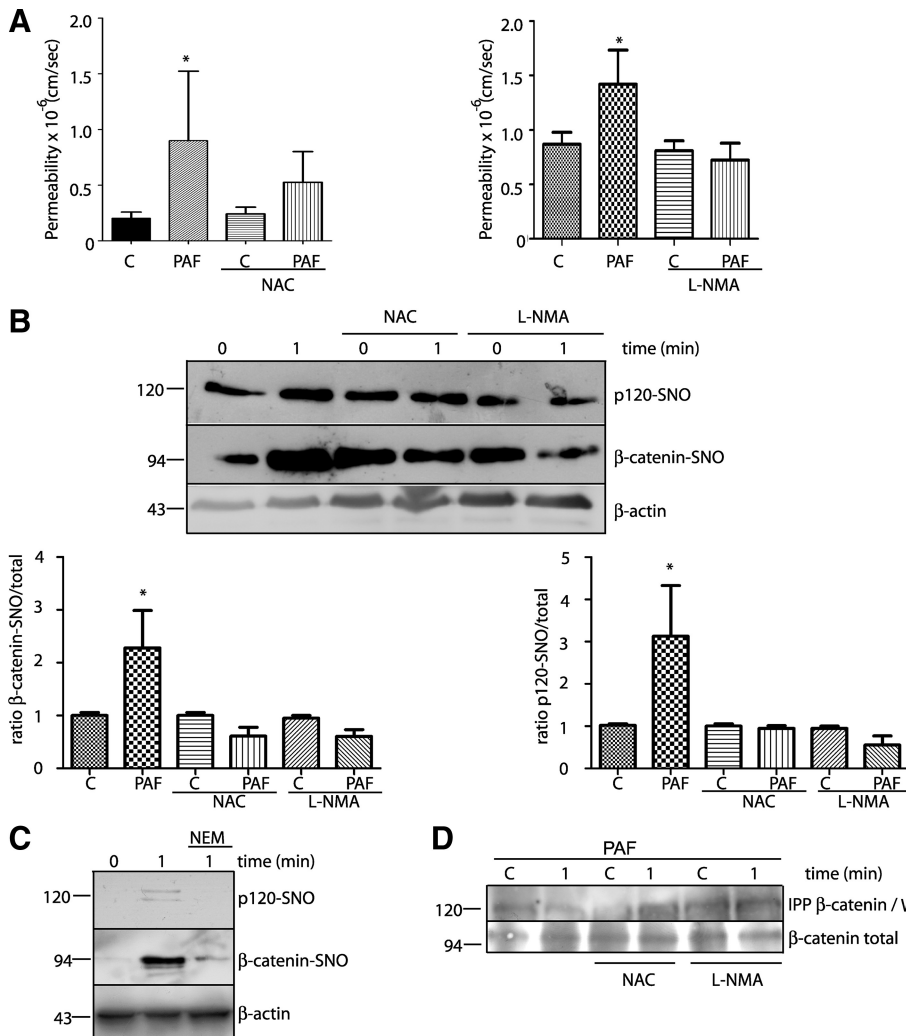


**Figure 5. S-nitrosation (SNO) disrupts the association between  $\beta$ -catenin and p120.** Protein extracts from ECV-GFPeNOS-G2A and ECV-GFPeNOS-CAAX cells, treated with  $5 \times 10^{-7}$  mol/L platelet-activating factor (PAF) for different times, were immunoprecipitated (IPP) with anti  $\beta$ -catenin antibodies and probed by Western blot with anti-p120 antibodies. **A**, Experiments in ECV-GFPeNOS-G2A cells. A representative Western blot assessing the association between  $\beta$ -catenin and p120 in ECV-GFPeNOS-G2A cells treated with PAF for different times is shown on the upper section, with corresponding statistical analysis illustrated below. Time indicates the duration of PAF application.  $*P < 0.05$  as compared with control;  $n = 3$ . **B**, Experiments in ECV-GFPeNOS-CAAX cells. The upper section shows a representative Western blot assessing whether or not PAF stimulates the disruption of the association between  $\beta$ -catenin and p120 in ECV-GFPeNOS-CAAX cells. The statistical evaluation is illustrated in the lower section. No statistical significance was observed.

the duration of PAF application.  $*P < 0.05$  as compared with control;  $n = 3$ . **B**, Experiments in ECV-GFPeNOS-CAAX cells. The upper section shows a representative Western blot assessing whether or not PAF stimulates the disruption of the association between  $\beta$ -catenin and p120 in ECV-GFPeNOS-CAAX cells. The statistical evaluation is illustrated in the lower section. No statistical significance was observed.

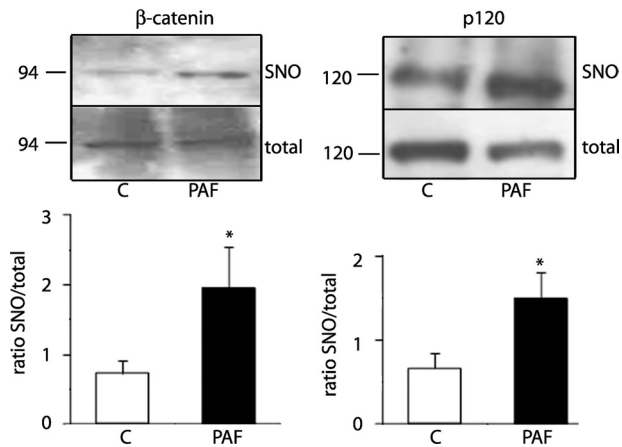
both NAC and L-NMA significantly inhibit PAF-induced hyperpermeability to FITC-dextran 70. The abolition of hyperpermeability was associated with an effective reduction of PAF-induced SNO of  $\beta$ -catenin and p120 in strong support of a cause-effect relationship between PAF-induced SNO and PAF-induced hyperpermeability to macromolecules (Figure

6B). Pretreatment of the cells for 15 minutes with 500  $\mu$ mol/L N-ethylmaleimide (which blocks sulfhydryl-groups impairing NO binding) strongly blocks SNO of  $\beta$ -catenin and p120 in response to PAF (Figure 6C). In further support of our hypothesis, we showed that NAC and L-NMA prevented the PAF-induced disruption of the association



**Figure 6. Platelet-activating factor (PAF)-induced hyperpermeability to macromolecules correlates strongly with S-nitrosation (SNO) of  $\beta$ -catenin and p120 in EAhy926 cells.** **A**, PAF-induced hyperpermeability in EAhy926 cells is blocked by inhibition of SNO with N-acetyl-L-cysteine (NAC; **left side**) and by inhibition of endothelial nitric oxide synthase (eNOS) with NG-methyl-L-arginine (L-NMA; **right side**). C=control. Data are expressed as mean permeability  $\pm$  SEM.  $*P < 0.05$  relative to control,  $n = 3$ . **B**, PAF-induced SNO of  $\beta$ -catenin and p120 is prevented by NAC, a competitive inhibitor of SNO, and by L-NMA, an inhibitor of eNOS.  $*P < 0.05$  as compared with control;  $n = 3$ . **C**, PAF-induced SNO of  $\beta$ -catenin and p120 is prevented by N-ethylmaleimide (NEM), a blocker of sulfhydryl groups. **D**, Protein extracts from EAhy926 cells treated with  $10^{-7}$  moles/L PAF for 1 minute, in the presence of NAC or L-NMA, were immunoprecipitated for  $\beta$ -catenin and probed by Western blot with anti-p120 antibodies. The representative blot shows that NAC and L-NMA blocked PAF-induced disruption of the association between  $\beta$ -catenin and p120.





**Figure 7. Platelet-activating factor (PAF) induces S-nitrosation (SNO) of  $\beta$ -catenin and p120 in the in vivo mouse cremaster muscle.** PAF, at  $10^{-7}$  moles/L, was applied to 1 cremaster muscle for 3 minutes while buffer was applied to the other cremaster (control) in the same animal. Homogenized tissues were prepared for biotin switch assay and streptavidin pull down to determine  $\beta$ -catenin and p120 SNO. The **top** panels show representative Western blot. The **lower** panels display the ratio of SNO to total protein (input) for each junctional protein. \* $P < 0.05$  as compared with control;  $n = 6$ .

between  $\beta$ -catenin and p120 (Figure 6C). Taken together, these data demonstrate SNO-mediated disruption of the interaction between  $\beta$ -catenin and p120 at the adherens junction is a relevant mechanism by which eNOS-derived NO regulates endothelial permeability.

**PAF Induces SNO In Vivo**

To assess whether or not SNO is a mechanism that causes hyperpermeability in vivo, we administered  $10^{-7}$  mol/L PAF to the mouse cremaster muscle and evaluated nitrosation by the biotin switch assay. PAF application resulted in significant SNO of  $\beta$ -catenin and p120 (Figure 7). The data, obtained at 3 minutes of continuous PAF application, are in agreement with previous reports demonstrating eNOS-derived NO induced hyperpermeability.<sup>1</sup>

**Identification of S-Nitrosated Cysteines in p120**

To determine the cysteines nitrosated by NO in p120, we exposed 1  $\mu$ g of purified p120 to 100  $\mu$ mol/L S-nitrosoglutathione at 37C

for 30 minutes (see Supplemental Data for a detailed account). Analysis by mass spectroscopy identified 5 cysteines that can be S-nitrosated: C429, C450, C579, C618, and C692 (Figure 8). Of these amino acids, C579 is the only residue that was S-nitrosated in 100% of the assayed peptides. C579 locates at helix 3 of repeat 5 of the Armadillo domain, a region that is important for the interactions of p120 with E-cadherin.<sup>42,43,28</sup> Therefore, the modification of this cysteine could affect the interaction with VE-cadherin.

**Discussion**

Our results advance the novel concept that location of eNOS is important for effective SNO of endothelial junctional proteins by NO, in particular  $\beta$ -catenin and p120. Our data provide strong support for the hypothesis that SNO of junctional proteins leads to hyperpermeability. Importantly, we show that SNO of  $\beta$ -catenin and p120 is a powerful mechanism that contributes to PAF-stimulated hyperpermeability in vivo.

Increases in microvascular permeability to macromolecules via paracellular pathways have been attributed to 2 main mechanisms: a) cytoskeletal contraction mediated by myosin light chain<sup>29</sup> and b) phosphorylation of adherens junction proteins that causes their internalization away from the adherens junctions.<sup>30,12</sup> These processes lead to reorganization of the adherens junctional complex and allow transport of macromolecules across endothelial monolayers. The process of permeability regulation is more complex as modifications of cyclic nucleotides by phosphodiesterases and changes in eNOS activity (leading to nitrosation and/or nitration) may play a role in reducing and enhancing the barrier properties of the endothelium.<sup>32,33</sup> Although nitric oxide has been reported as an agent that contributes to decrease microvascular permeability<sup>34</sup> as well as to increase it,<sup>3,35</sup> the importance of NO in promoting the onset of endothelial permeability in response to proinflammatory agents has been unequivocally demonstrated using eNOS knockout mouse<sup>1,36</sup> and cells treated with siRNA for eNOS.<sup>2</sup>

There are no reports of direct effects of NO in the adherens junction in response to PAF in the literature. Using 3 different cell models (postcapillary venular endothelial cells, EAhy926 and ECV-eNOSGFP cells), we demonstrate SNO of

1	MDDSEVESTA	SILASVKEQE	AQFEKLTAL	EEERRHVSQA	LERVRVSPQD	ANPLMANGTL
61	TRRHQNGRFV	GDADLERQKF	SDLKLNQPQD	HSLLLYSTIP	RMQEPGQIVE	TYTEEDPEGA
121	MSVSVVETSD	DGTRTRTETT	VKKVVKTVT	RTVQPVAMGP	DGLPVDASSV	SNNYIQTLLGR
181	DFRKNNGGGP	GPYVQGAGTA	TLPRNFHYPP	DGYSRHYEDG	YPGGSDNYGS	LSRVTRIEER
241	YRPSMEGYRA	PSRQDVYGPQ	PQVRVGGSSV	DLHRFHPEPY	GLEDDQSRMG	YDDLQYGMMS
301	DYGTARTTGT	PSDPRRLRS	YEDMIGEVP	SDQYIWAPLA	QHERGSLASL	DSLKGGPPFP
361	<b>PNWRQPELPE</b>	<b>VIAMLGFRLD</b>	<b>AVKSNAAYL</b>	<b>QHLCYRNDRV</b>	<b>KTDVRLKLGKI</b>	<b>PVLVGLLDHP</b>
421	<b>KKEVHLGAG</b>	<b>ALKNISFGRD</b>	<b>QDNKIAIKNC</b>	<b>DGVPALVRL</b>	<b>RKARDMDLTE</b>	<b>VITGTLWNLS</b>
481	<b>SHDSIKMEIV</b>	<b>DHALHALTDE</b>	<b>VIIPHSGNER</b>	<b>EPNEDCKPRH</b>	<b>IEWESVLTNT</b>	<b>AGCLRNVSE</b>
541	<b>RSEARRKLR</b>	<b>CDGLVDALIF</b>	<b>IVQAEIGQKD</b>	<b>SDSKLVENCV</b>	<b>CLLRNLSYQV</b>	<b>HREIPQAERY</b>
601	<b>QEAAPNVANN</b>	<b>TGPHAAFCFG</b>	<b>AKGKDEWFS</b>	<b>RGKKPIEDPA</b>	<b>NDTVDFPKRT</b>	<b>SPARGYELLF</b>
661	QPEVVRIYIS	LLKESKTPAI	LEASAGAIQN	<b>L</b> CAGRWTYGR	YIRSALRQEK	ALSAIADLLT
721	NEHERVVKAA	SGALRNLAVD	ARNKELIGKH	AIPNLVKNLP	GGQQNSSWNF	SEDTVISILN
781	TINEVIAENL	EAAKLRRTQ	GIKLVLINK	SGNRSEKEVR	AAALVLQTIW	GYKELRKPFE
841	KEGWKKSDFQ	VNLNNASRSQ	SSHSDYDSTL	PLIDRNQKSD	KKPDREEIQM	SNMGSNTKSL
901	DNNYSTPNER	GDHNRTLDRS	GDLGDMEPLK	GTTPLMQDEG	QESLEELLDV	LVLDEGGQV
961	SYPSMQKI					

**Figure 8. Mapping of S-nitrosation sites in p120.** Protein sequence from human p120. Purified p120 was S-nitrosated with S-nitrosoglutathione for 30 minutes, subjected to biotin switch assay followed by in-solution trypsin digest. The region that potentially interacts with vascular endothelial-cadherin is shown in gray background. Cysteine 579 (white letter, red background) was 100% S-nitrosated according to mass spectrometry. Other cysteines (red background) were not S-nitrosated in 100% of the assays. Unlabeled cysteines were not S-nitrosated. (See online-only Supplemental Data for experimental details.)

$\beta$ -catenin and p120 in response to PAF. This modification leads to changes in the localization and interaction between these proteins at the adherens junction, which finally leads to hyperpermeability. This is the first report linking NO signaling directly to p120 as a target at the adherens junction. Furthermore, we demonstrate that this mechanism does occur in vivo as PAF caused SNO of  $\beta$ -catenin and p120 in the mouse cremaster muscle at times corresponding to the onset of the hyperpermeability response.

The classic dogma in NO signaling establishes that the actions of NO are mediated via sGC and PKG. However, increases in permeability occur in some cellular models regardless of PKG activation but still require NO production.<sup>14</sup> Our results after inhibition of sGC with ODQ agree with the findings that synthesis of cGMP and activation of PKG are not essential for the development of hyperpermeability in response to PAF (Figure 3). Recently, SNO has emerged as an important NO-dependent posttranslational modification (independent of sGC/PKG pathway) of free-thiol cysteines that alters the function of proteins, affecting processes of intracellular trafficking and phosphorylation and requires proximity between eNOS and the target proteins for appropriate NO delivery.<sup>15,16,17</sup> Our results showing SNO of  $\beta$ -catenin and p120 in cells with cytosolic eNOS strongly confirm the requirement of proximity to induce the modification. The fact that plasma membrane-anchored eNOS fails to induce SNO of these proteins despite that it efficiently produces NO strongly supports the concept that SNO depends on NO local concentration and the relevance of directed SNO as a mechanism to increase permeability. In support of these concepts, we demonstrate that acetylcholine—an agonist that localizes eNOS to Golgi, stimulates production of NO and induces vasodilation<sup>24,37,23</sup>—does not cause SNO. PAF may cause enhanced permeability by formation of peroxynitrite,<sup>38</sup> the reaction product of NO and superoxide anion.<sup>39</sup> In the hamster cheek pouch, 3-morpholinopyridone N-ethylcarbamide,<sup>40</sup> a peroxynitrite generator, induces hyperpermeability.<sup>41</sup> However, we did not pursue this line of inquiry because peroxynitrite preferentially leads to protein nitration;<sup>42</sup> ie, protein modification via NO binding to tyrosine rather than by protein nitrosation of cysteines.

Our combined data make a compelling case for SNO of key junctional proteins induced by eNOS-derived NO as a novel mechanism for regulation of permeability to macromolecules across endothelial cell monolayers and the microvasculature. Dissociation between  $\beta$ -catenin and p120 from VE-cadherin disrupts the junctional complex and leads to loss of barrier integrity and enhanced transport of molecules across endothelial monolayers and/or postcapillary venules.<sup>31</sup> We focused on  $\beta$ -catenin and p120 as relevant targets. The interactions of  $\beta$ -catenin and p120 with VE-cadherin are required for the barrier function. In fact, the absence of p-120<sup>43</sup> and  $\beta$ -catenin<sup>44,45,43</sup> causes loss of barrier integrity and an increase in permeability even in the presence of VE-cadherin. Recently, SNO of  $\beta$ -catenin was associated with changes in permeability in response to 15-minute application of vascular endothelial growth factor.<sup>46</sup> While confirming SNO of  $\beta$ -catenin, we demonstrate the novel observation

that SNO occurs at early times that are consistent with the onset of hyperpermeability in response to proinflammatory agonists. In addition, we provide novel evidence showing that SNO of p120 is an important molecular mechanism in PAF-induced hyperpermeability. The relationships and interactions between SNO and phosphorylation, an established regulatory mechanism, remain to be elucidated. Recent evidence suggests that SNO plays an important role because mutation of C619, which is subject to SNO, was sufficient to inhibit Vascular endothelial growth factor-induced hyperpermeability even though phosphorylation increased in response to the agonist.<sup>41</sup> In the case of p120 the phosphorylation sites are in the regulatory domain and tail domain, whereas the cysteines that are available for SNO are in the region of possible interactions between p120–VE-cadherin. From these observations, it is possible to speculate that parallel synergism may exist between phosphorylation and SNO in the process of internalization of junctional proteins.

Based on our mass spectrometry results, we propose that C579 is a strong candidate for SNO-regulation by NO in vivo because it is located in the molecular stretch that interacts with E-cadherin.<sup>47,48</sup> Because of the high homology between E-cadherin and VE-cadherin, it is plausible that SNO of this cysteine affects the interactions of p120 with VE-cadherin. Modification of cysteines by NO is a new chemical mechanism that alters protein-protein interactions at the cell junction and causes enhanced permeability.

Our data demonstrating SNO of  $\beta$ -catenin and p120 in response to PAF and TNF- $\alpha$  confirm and expand the observation that eNOS-derived NO causes nitrosation of  $\beta$ -catenin in response to VEGF.<sup>46</sup> These combined data suggest that SNO of junctional proteins may be a universal mechanism shared by proinflammatory agonists. Importantly, we advance the field by demonstrating that SNO of junctional proteins occurs at times compatible with the onset of hyperpermeability. The NO modification of the cysteines is a new mechanism that alters protein-protein interactions at the cell junction and causes enhanced permeability.

## Sources of Funding

This work was supported by grant FONDECYT 1100569, Anillos-ACT71, and NIH grants 5R01 HL070634 and 5R01 HL088479. The mass spectrometry data were obtained using an Orbitrap instrument funded in part by NIH grant NS046593 for the support of the University of Medicine and Dentistry of New Jersey's Neuroproteomics Core Facility.

## Disclosures

None.

## References

1. Hatakeyama T, Pappas PJ, Hobson RW, II, Boric MP, Sessa WC, Duran WN. Endothelial nitric oxide synthase regulates microvascular hyperpermeability in vivo. *J Physiol (Lond)*. 2006;574:275–281.
2. Sanchez FA, Kim DD, Duran RG, Meininger CJ, Duran WN. Internalization of eNOS via caveolae regulates PAF-induced inflammatory hyperpermeability to macromolecules. *Am J Physiol Heart Circ Physiol*. 2008;295:H1642–H1648.

3. Ramirez MM, Quardt SM, Kim D, Oshiro H, Minnicozzi M, Duran WN. Platelet activating factor modulates microvascular permeability through nitric oxide synthesis. *Microvasc Res*. 1995;50:223–234.
4. Yuan SY. New insights into eNOS signaling in microvascular permeability. *Am J Physiol Heart Circ Physiol*. 2006;291:H1029–H1031.
5. Mayhan WG. Role of nitric oxide in modulating permeability of hamster cheek pouch in response to adenosine 5'-diphosphate and bradykinin. *Inflammation*. 1992;16:295–305.
6. Sanchez FA, Rana R, Gonzalez FG, Iwahashi T, Duran RG, Fulton DJ, Beuve AV, Kim DD, WN Duran. Functional significance of cytosolic endothelial nitric-oxide synthase (eNOS): regulation of hyperpermeability. *J Biol Chem*. 2011;286:30409–30414.
7. Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev*. 2004;84:869–901.
8. Dejana E. Endothelial adherens junctions: implications in the control of vascular permeability and angiogenesis. *J Clin Invest*. 1996;98:1949–1953.
9. Duran WN. The double-edge sword of TNF-alpha in ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2008;295:H2221–H2222.
10. Geiger B, Ayalon O. Cadherins. *Annu Rev Cell Biol*. 1992;8:307–332.
11. Weis WI, Nelson WJ. Re-solving the cadherin-catenin-actin conundrum. *J Biol Chem*. 2006;281:35593–35597.
12. Esser S, Lampugnani MG, Corada M, Dejana E, Risau W. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci*. 1998;111:1853–1865.
13. Shasby DM, Ries DR, Shasby SS, Winter MC. Histamine stimulates phosphorylation of adherens junction proteins and alters their link to vimentin. *Am J Physiol Lung Cell Mol Physiol*. 2002;282:L1330–L1338.
14. Lakshminarayanan S, Antonetti DA, Gardner TW, Tarbell JM. Effect of VEGF on retinal microvascular endothelial hydraulic conductivity: the role of NO. *Invest Ophthalmol Vis Sci*. 2000;41:4256–4261.
15. Iwakiri Y, Satoh A, Chatterjee S, Toomre DK, Chalouni CM, Fulton D, Groszmann RJ, Shah VH, Sessa WC. Nitric oxide synthase generates nitric oxide locally to regulate compartmentalized protein S-nitrosylation and protein trafficking. *Proc Natl Acad Sci U S A*. 2006;103:19777–19782.
16. Huang Y, Man HY, Sekine-Aizawa Y, Han Y, Juluri K, Luo H, Cheah J, Lowenstein C, Hagan RL, Snyder SH. S-nitrosylation of N-ethylmaleimide sensitive factor mediates surface expression of AMPA receptors. *Neuron*. 2005;46:533–540.
17. Ozawa K, Whalen EJ, Nelson CD, Mu Y, Hess DT, Lefkowitz RJ, Stamler JS. S-nitrosylation of beta-arrestin regulates beta-adrenergic receptor trafficking. *Mol Cell*. 2008;31:395–405.
18. Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE*. 2001;2001:11.
19. Aramoto H, Breslin JW, Pappas PJ, Hobson RW, II, Duran WN. Vascular endothelial growth factor stimulates differential signaling pathways in vivo microcirculation. *Am J Physiol Heart Circ Physiol*. 2004;287:H1590–H1598.
20. Xiao K, Allison DF, Kottke MD, Summers S, Sorescu GP, Faundez V, Kowalczyk AP. Mechanisms of VE-cadherin processing and degradation in microvascular endothelial cells. *J Biol Chem*. 2003;278:19199–19208.
21. Sanchez FA, Rana R, Kim DD, Iwahashi T, Zheng R, Lal BK, Gordon DM, Meininger CJ, Duran WN. Internalization of eNOS and NO delivery to subcellular targets determine agonist-induced hyperpermeability. *Proc Natl Acad Sci U S A*. 2009;106:6849–6853.
22. Meininger CJ, Schelling ME, Granger HJ. Adenosine and hypoxia stimulate proliferation and migration of endothelial cells. *Am J Physiol*. 1988;255:H554–H562.
23. Sanchez FA, Savalia NB, Duran RG, Lal BK, Boric MP, Duran WN. Functional significance of differential eNOS translocation. *Am J Physiol Heart Circ Physiol*. 2006;291:H1058–H1064.
24. Kim DD, Kanetaka T, Duran RG, Sanchez FA, Bohlen HG, Duran WN. Independent regulation of periaarteriolar and perivenular nitric oxide mechanisms in the in vivo hamster cheek pouch microvasculature. *Microcirculation*. 2009;16:323–330.
25. Erwin PA, Lin AJ, Golan DE, Michel T. Receptor-regulated dynamic S-nitrosylation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem*. 2005;280:19888–19894.
26. Fulton D, Babbitt R, Zoellner S, Fontana J, Acevedo L, McCabe TJ, Iwakiri Y, Sessa WC. Targeting of endothelial nitric-oxide synthase to the cytoplasmic face of the Golgi complex or plasma membrane regulates Akt- versus calcium-dependent mechanisms for nitric oxide release. *J Biol Chem*. 2004;279:30349–30357.
27. Sessa WC, Garcia-Cardena G, Liu J, Keh A, Pollock JS, Bradley J, Thiru S, Braverman IM, Desai KM. The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. *J Biol Chem*. 1995;270:17641–17644.
28. Ishiyama N, Lee SH, Liu S, Li GY, Smith MJ, Reichardt LF, Ikura M. Dynamic and static interactions between p120 catenin and E-cadherin regulate the stability of cell-cell adhesion. *Cell*. 2010;141:117–128.
29. Yuan SY. Signal transduction pathways in enhanced microvascular permeability. *Microcirculation*. 2000;7:395–403.
30. Alexander JS, Alexander BC, Eppihimer LA, Goodyear N, Haque R, Davis CP, Kalogeris TJ, Carden DL, Zhu YN, Kevil CG. Inflammatory mediators induce sequestration of VE-cadherin in cultured human endothelial cells. *Inflammation*. 2000;24:99–113.
31. Kevil CG, Payne DK, Mire E, Alexander JS. Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins. *J Biol Chem*. 1998;273:15099–15103.
32. Rentsendorj O, Damarla M, Aggarwal NR, Choi JY, Johnston L, D'Alessio FR, Crow MT, Pearce DB. Knockdown of lung phosphodiesterase 2A attenuates alveolar inflammation and protein leak in a two-hit mouse model of acute lung injury. *Am J Physiol Lung Cell Mol Physiol*. 2011;L161–L170.
33. Siddiqui MR, Komarova YA, Vogel SM, Gao X, Bonini MG, Rajasingh J, Zhao YY, Brovkovich V, Malik AB. Caveolin-1-eNOS signaling promotes p190RhoGAP-A nitration and endothelial permeability. *J Cell Biol*. 193:841–850.
34. Kubes P, Granger DN. Nitric oxide modulates microvascular permeability. *Am J Physiol*. 1992;262:H611–H615.
35. Yuan Y, Granger HJ, Zawieja DC, DeFily DV, Chilian WM. Histamine increases venular permeability via a phospholipase C-NO synthase-guanylate cyclase cascade. *Am J Physiol*. 1993;264:H1734–H1739.
36. Fukumura D, Gohongi T, Kadambi A, Izumi Y, Ang J, Yun CO, Buerk DG, Huang PL, Jain RK. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. *Proc Natl Acad Sci U S A*. 2001;98:2604–2609.
37. Figueroa XF, Gonzalez DR, Martinez AD, Duran WN, Boric MP. ACh-induced endothelial NO synthase translocation, NO release and vasodilatation in the hamster microcirculation in vivo. *J Physiol (Lond)*. 2002;544:883–896.
38. Klabunde RE, Anderson DE. Role of nitric oxide and reactive oxygen species in platelet-activating factor-induced microvascular leakage. *J Vasc Res*. 2002;39:238–245.
39. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A*. 1990;87:1620–1624.
40. Dairou J, Atmane N, Rodrigues-Lima F, Dupret JM. Peroxynitrite irreversibly inactivates the human xenobiotic-metabolizing enzyme arylamine N-acetyltransferase 1 (NAT1) in human breast cancer cells: a cellular and mechanistic study. *J Biol Chem*. 2004;279:7708–7714.
41. González F. Participación Del Óxido Nítrico En La Hiperpermeabilidad A Macromoléculas Inducida Por El Factor Activador De Plaquetas. Acción Del Óxido Nítrico Sobre Distintas Vías De Señalización. Santiago, Chile: Pontificia Universidad Católica de Chile; 2009.
42. Xiong Y, Rabchevsky AG, Hall ED. Role of peroxynitrite in secondary oxidative damage after spinal cord injury. *J Neurochem*. 2007;100:639–649.
43. Herron CR, Lowery AM, Hollister PR, Reynolds AB, Vincent PA. p120 regulates endothelial permeability independently of its NH2 terminus and Rho binding. *Am J Physiol Heart Circ Physiol*. 2011;300:H36–H48.
44. Liebner S, Corada M, Bangsow T, et al. Wnt/beta-catenin signaling controls development of the blood-brain barrier. *J Cell Biol*. 2008;183:409–417.
45. Cattellino A, Liebner S, Gallini R, Zanetti A, Balconi G, Corsi A, Bianco P, Wolburg H, Moore R, Oreda B, Kemler R, Dejana E. The conditional inactivation of the beta-catenin gene in endothelial cells causes a defective vascular pattern and increased vascular fragility. *J Cell Biol*. 2003;162:1111–1122.



46. Thibeault S, Rautureau Y, Oubaha M, Faubert D, Wilkes BC, Delisle C, Gratton JP. S-nitrosylation of beta-catenin by eNOS-derived NO promotes VEGF-induced endothelial cell permeability. *Mol Cell*. 2010;39:468–476.
47. Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy F, Reynolds AB. A novel role for p120 catenin in E-cadherin function. *J Cell Biol*. 2002;159:465–476.
48. Liu H, Komiya S, Shimizu M, Fukunaga Y, Nagafuchi A. Involvement of p120 carboxy-terminal domain in cadherin trafficking. *Cell Struct Funct*. 2007;32:127–137.

## Novelty and Significance

### What Is Known?

- Nitric oxide is a key factor in regulating microvascular permeability.
- Platelet-activating factor (PAF) increases permeability via translocation of endothelial nitric oxide synthase to cytosol.
- Increases in permeability can be stimulated independently of soluble guanylyl cyclase-protein kinase G activation.

### What New Information Does This Article Contribute?

- S-nitrosation of p120-catenin and  $\beta$ -catenin increases endothelial permeability to macromolecules.
- Increase in permeability in response to PAF can occur independently of soluble guanylyl cyclase-protein kinase G activation.
- PAF induced S-nitrosation of junctional proteins  $\beta$ -catenin and p120 and their internalization away from the plasma membrane.
- Endothelial nitric oxide synthase located in the cytosol, but not plasma membrane-anchored endothelial nitric oxide synthase, causes S-nitrosation of  $\beta$ -catenin and p120.
- S-nitrosation of  $\beta$ -catenin and p120 significantly diminished association between these proteins.
- Mass spectrometry analysis of purified p120 identified cysteine 579 as the main S-nitrosated residue.

During acute inflammation many mediators act on endothelial cells increasing microvascular permeability. Endothelial adherens junctions constitute an important element in the control of microvascular permeability. The mechanisms by which nitric oxide signaling regulates permeability at adherens junctions are still incompletely understood. We demonstrate S-nitrosation of  $\beta$ -catenin and p120 in response to PAF. This modification leads to changes in the localization and interaction between these proteins at the adherens junction, which leads to hyperpermeability. This is the first report linking nitric oxide signaling directly to p120 as a target at the adherens junction. Furthermore, we demonstrate that this mechanism does occur *in vivo* as PAF caused S-nitrosation of  $\beta$ -catenin and p120 in the mouse cremaster muscle at times corresponding to the onset of the increase in permeability. We identify cysteine 579 in p120 as the main residue S-nitrosated during the increase in permeability. We anticipate that our results demonstrating the significance of S-nitrosation in regulation of microvascular permeability may serve as a basis for the development of new therapeutic strategies in the treatment of vascular diseases characterized by inflammation.



## SUPPLEMENTAL MATERIAL

### Cells used in our study:

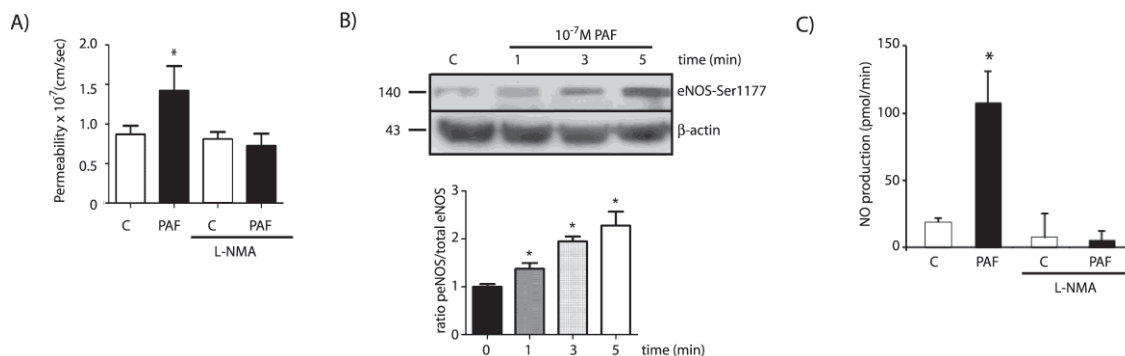
**Coronary venular endothelial cells (CVEC).** These cells (obtained from bovine hearts<sup>1</sup>) are derived from postcapillary venules, the main site of blood-tissue transport of macromolecules. This microvascular segment constitutes the main target for inflammatory agents, and, therefore, provides a clinically relevant correlation.

**EAhy926 cells.** This human umbilical vein cell line was established by fusing primary human umbilical vein cells with a thioguanine-resistant clone of A549 by exposure to polyethylene glycol<sup>2</sup>. Hybrid clones were selected in HAT medium and screened for factor VIII-related antigen. EAhy926 cells show characteristics of differentiated endothelial cell functions such as angiogenesis, homeostasis/thrombosis, blood pressure and inflammation<sup>2</sup>.

**ECV-304 cells.** ECV-304 cells were based originally on human umbilical vein endothelial cells and became greatly modified, including loss of eNOS, due to contamination with epithelial cells from a human urinary bladder carcinoma<sup>3, 4</sup>. We chose ECV-304 cells for this study because they resemble endothelial cells functionally but do not express endogenous eNOS<sup>5</sup>. ECV-304 cells were transfected with eNOS-GFP and with eNOS-GFP constructs that target eNOS to either the plasma membrane (GFP-eNOS-CAAX) or the cytosol (GFP-eNOS-G2A)<sup>6, 7</sup>. These conditions allowed us to activate eNOS and to study unequivocally the functional impact of its subcellular location.

### Endothelial monolayer permeability and EAhy926 cells:

Online Figure IA shows the permeability response of confluent EAhy926 cells to  $1 \times 10^{-7}$  moles/L PAF. As shown, PAF induced a significant increase in permeability from  $0.87 \pm 0.06 \times 10^{-6}$  cm/sec to  $1.42 \pm 0.18 \times 10^{-6}$  cm/sec. This increase was inhibited by N-Methyl-L-arginine acetate (L-NMA), an inhibitor of eNOS. Online Figure IB illustrates baseline and demonstrates significant PAF-stimulated phosphorylation of eNOS at Ser1177 in EAhy926 cells. PAF-stimulated phosphorylation of eNOS increased at 30 seconds and reached at maximum at 3 minutes. Phosphorylation of eNOS was associated with NO production (Online Figure IC). These data demonstrate that EAhy926 cells are an appropriate cell model for the study of regulatory mechanisms of endothelial permeability.

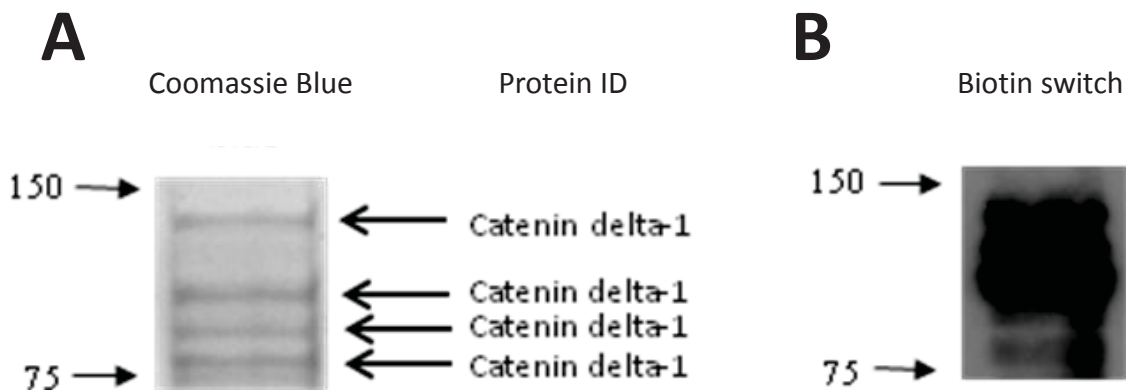


**Online Figure I.** PAF stimulates hyperpermeability to macromolecules in EAhy926 cells through eNOS activation. A) PAF increases permeability to FITC-dextran-70 across confluent EAhy926 monolayers. PAF-stimulated hyperpermeability is abolished by inhibition of eNOS with L-N-methyl arginine (L-NMA). C = control. B) Western blot demonstrating that  $10^{-7}$  moles/L PAF causes phosphorylation of eNOS on serine 1177 (Ser1177).  $\beta$ -actin served as loading control. C) PAF stimulates NO production, while L-NMA inhibits PAF-stimulated NO production.

Determination of S-nitrosation of p120 by mass spectrometry:

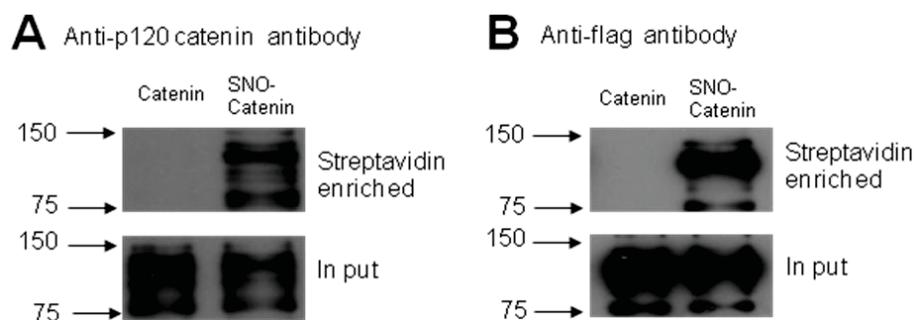
**SDS separation and protein identification of p120, and Biotin Switch Western blotting of p120-SNO.** To confirm the purity of the recombinant human p120 protein, 0.5 µg proteins were separated using 10% SDS gel and coomassie blue stain. The gel showed four bands between 75 kDa -150 kDa MW. The bands were cut and digested with trypsin. Protein identification was performed using 4800 MALDI TOF/TOF™ Analyzer (Applied Biosystems, Carlsbad, California USA). The results identified the four bands as human p120 (catenin delta 1, CTND1\_HUMAN) (Online Figure IIA).

To detect the S-nitrosylation level of the protein, we exposed 1 µg recombinant p120 to 100 µM S-Nitrosoglutathione (GSNO) at 37C for 30 min. The proteins were then precipitated with 4x ice cold acetone (50 µg lysozyme as a carrier), washed with ice cold acetone, and assessed by biotin switch and western blotting. Free thiols were blocked with 20 mM MMTS followed by acetone precipitation and washed with 80% ice cold acetone (3x). S-nitrosated cysteines were reduced with 10 mM ascorbate and labeled with biotin HPDP. Following biotin switch, Western blotting was performed to probe against biotin. About 0.5 µg of protein was separated using nonreducing SDS-PAGE and transferred onto nitrocellulose membranes. The biotinylated protein was probed with an anti-biotin antibody (1:3,000) (Vector Laboratories, Burlingame, CA) and visualized with enhanced chemiluminescent substrate (PerkinElmer Life Science) (Online Figure IIB).



**Online Figure II.** SDS separation and protein identification of p120 and Biotin switch Western blotting of p120-SNO. 0.5 µg proteins were separated using 10% SDS gel and the gel was stained with coomassie blue, four bands were cut and performed for trypsin digestion and protein identification using. The result showed that four bands are all human catenin delta 1 (CTND1\_HUMAN) (Fig.II A). The biotinylated sample was performed for Western blotting. About 0.5 µg of protein was separated using nonreducing SDS-PAGE and transferred onto nitrocellulose membranes. The biotinylated protein was probed with an anti-biotin antibody (1:3,000) (Vector Laboratories, Burlingame, CA) and visualized with enhanced chemiluminescent substrate (PerkinElmer Life Science) (Fig. II B).

**Avidin enrichment and Western blotting of p120-SNO.** In order to confirm the S-nitrosylation of p120, biotinylated samples (about 2  $\mu\text{g}$  catenin in 10  $\mu\text{l}$  buffer) were enriched using 5  $\mu\text{l}$  streptavidin agarose resin (Thermo Scientific, Hudson, New Hampshire USA). The enriched biotinylated p120 was eluted using 2x SDS gel sample buffer at 100C for 5 min, separated using 10% SDS gel and transferred onto nitrocellulose membranes. The enriched protein was probed with an anti-p120 antibody (1:2500) (BD Bioscience, Sparks, MD, USA) and visualized with enhanced chemiluminescent substrate (PerkinElmer Life Science) (Online Figure IIIA). The membrane was reprobred using anti-flag antibody (1:2000) (p120 is flag tagged) to confirm the result (Online Figure IIIB).



**Online Figure III.** Avidin enrichment and Western blotting of SNO p120catenin. Panel A: The enriched protein was probed with an anti-p120-catenin antibody. Panel B. The membrane was reprobred using anti-flag antibody.

**Identification of S-nitrosated peptides in p120-SNO.** Biotinylated sample (about 0.1  $\mu\text{g}$  p120-catenin) was resolved in 8 M urea after acetone precipitation, diluted 10 times using 50 mM  $\text{NH}_4\text{HCO}_3$  and digested with 0.01  $\mu\text{g}$  trypsin. The peptides were desalted with a  $\text{C}_{18}$  ziptip, and identified by Orbitrap Velos MS. The MS/MS spectra were searched against a Swissprot human database using Thermo proteome Discoverer 1.3.0.339 software with biotin HPDP as a variable modification. Peptides identified with 95% confidence interval were displayed in Scaffold3 software. Protein and peptide False Discovery Rates were less than 1%. (Online Fig 4 –8; Tables I and II). In Tables I and II, if one cysteine in one peptide was labeled by MMTS and biotin HPDP, it means the cysteine was not 100% S-nitrosated.

**Table I. Peptides identified with biotinylated cysteines in SNO p120-catenin**

Sequence	Modifications
(K)EVHLGAcGALK(N)	Biotin-HPDP (+428)
(K)LVENCVC*LLR(N)	Biotin-HPDP (+428), MMTS (+46)
(K)NcDGVPALVR(L)	Biotin-HPDP (+428)
(K)TPAILEASAGAIQNLcAGR(W)	Biotin-HPDP (+428)
(R)YQEAAPNVANNTGPHAAScFGAK(K)	Biotin-HPDP (+428)

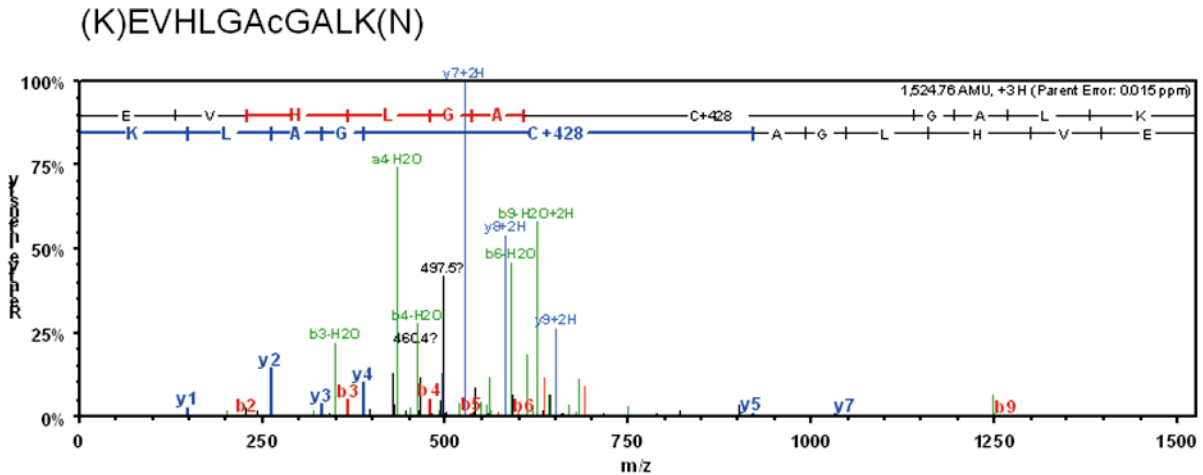
Note : **c** Biotin-HPDP (+428), It means the cysteine was S-nitrosated

**C\*** MMTS (+46), it means the cysteine had free thiol that had not been s- nitrosated.

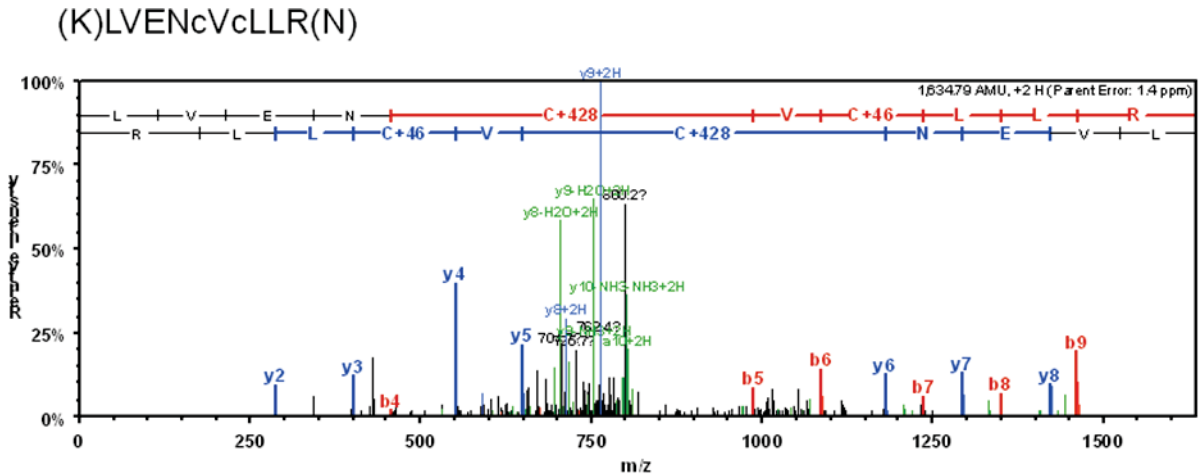
**Table II. Identified peptides with MMTS labeled cysteines in SNO p120-catenin**

Sequence	Modifications
(K)EVHLGAcGALK(N)	MMTS (+46)
(R)HIEWESVLTNTAG C*LR(N)	MMTS (+46)
(K)KEVHLGA C*GALK(N)	MMTS (+46)
(K)LVENCv C*LLR(N)	Biotin-HPDP (+428), MMTS (+46)
(K)N C*DGVPALVR(L)	MMTS (+46)
(K)SNAAAYLQHL C*YR(N)	MMTS (+46)
(K)TPAILEASAGAIQNL C*AGR(W)	MMTS (+46)
(R)YQEAAPNVANNTGPHAAS C*FGAK(K)	MMTS (+46)

Note : c Biotin-HPDP (+428), It means the cysteine was S-nitrosated. C\* MMTS (+46), it means the cysteine had free thiol that had not been S-nitrosated.



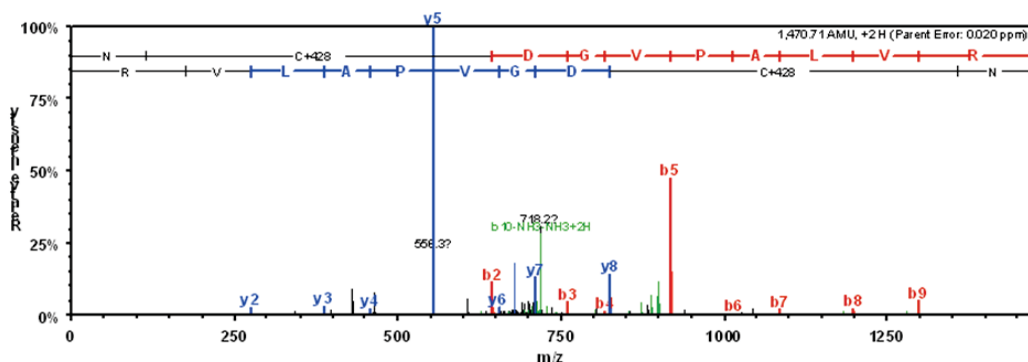
**Online Figure IV.** MS/MS spectrum evidence showing that the peptide EVHLGAcGALK in p120 is biotinylated, which indicates S-nitrosation. c: biotin HPDP labeled (S-nitrosated).



**Online Figure V.** MS/MS spectrum evidence showing that the peptide LVENCvLLR in p120 is biotinylated, which demonstrates S-nitrosation. c: biotin HPDP labeled (S-nitrosated), C :MMTS labeled.

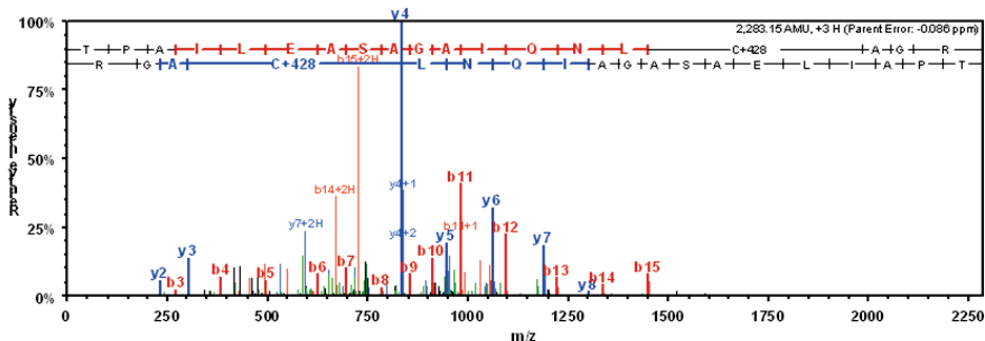


(K)NcDGVPALVR(L)



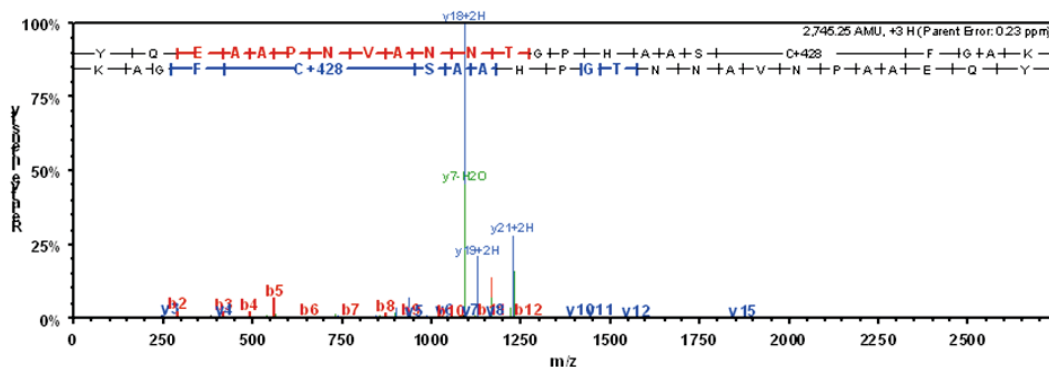
Online Figure VI. MS/MS spectrum evidence showing that the peptide NcDGVPALVR in p120 is biotinylated which demonstrates S-nitrosation. c: biotin HPDP labeled (S-nitrosated).

(K)TPAILEASAGAIQNLcAGR(W)



Online Figure VII. MS/MS spectrum evidence showing that the peptide TPAILEASAGAIQNLcAGR in p120 is biotinylated, which demonstrates S-nitrosation. c: biotin HPDP labeled (S-nitrosated).

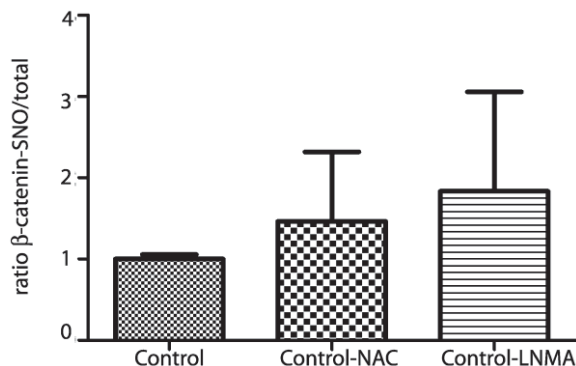
(R)YQEAAPNVANNTGPHAAScFGAK(K)



Online Figure VIII. MS/MS spectrum evidence showing that the peptide YQEAAPNVANNTGPHAAScFGAK in p120 is biotinylated, which demonstrates S-nitrosation. c: biotin HPDP labeled (S-nitrosated).

### NAC and L-NMA do not significantly reduce control S-nitrosation of $\beta$ -catenin.

We quantified the control levels of  $\beta$ -catenin-SNO in the presence of NAC and L-NMA in EAhy926 cells. Online Figure IX shows that pre-treatment of control cells with NAC and L-NMA does not significantly modify the S-nitrosation levels of  $\beta$ -catenin.



**Online Figure IX.** Quantification of control  $\beta$ -catenin-SNO in the presence of NAC and L-NMA.

## REFERENCES

1. Schelling ME, Meininger CJ, Hawker JR, Jr., Granger HJ. Venular endothelial cells from bovine heart. *The American journal of physiology*. 1988;254:H1211-1217.
2. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proceedings of the National Academy of Sciences of the United States of America*. 1983;80:3734-3737.
3. Brown J, Reading SJ, Jones S, Fitchett CJ, Howl J, Martin A, Longland CL, Michelangeli F, Dubrova YE, Brown CA. Critical evaluation of ECV304 as a human endothelial cell model defined by genetic analysis and functional responses: a comparison with the human bladder cancer derived epithelial cell line T24/83. *Lab Invest*. 2000;80:37-45.
4. Drexler HG, Quentmeier H, Dirks WG, MacLeod RA. Bladder carcinoma cell line ECV304 is not a model system for endothelial cells. *In Vitro Cell Dev Biol Anim*. 2002;38:185-186; author reply 187.
5. Sanchez FA, Savalia NB, Duran RG, Lal BK, Boric MP, Duran WN. Functional significance of differential eNOS translocation. *American journal of physiology. Heart and circulatory physiology*. 2006;291:H1058-1064.
6. Sowa G, Liu J, Papapetropoulos A, Rex-Haffner M, Hughes TE, Sessa WC. Trafficking of endothelial nitric-oxide synthase in living cells. Quantitative evidence supporting the role of palmitoylation as a kinetic trapping mechanism limiting membrane diffusion. *The Journal of biological chemistry*. 1999;274:22524-22531.
7. Church JE, Fulton D. Differences in eNOS activity because of subcellular localization are dictated by phosphorylation state rather than the local calcium environment. *The Journal of biological chemistry*. 2006;281:1477-1488.